Intranasal deferoxamine reverses iron-induced memory deficits and inhibits amyloidogenic APP processing in a transgenic mouse model of Alzheimer’s disease

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Abstract

Increasing evidence indicates that a disturbance of normal iron homeostasis and an amyloid-\(\beta\) (A\(\beta\))-iron interaction may contribute to the pathology of Alzheimer’s disease (AD), whereas iron chelation could be an effective therapeutic intervention. In the present study, transgenic mice expressing amyloid precursor protein (APP) and presenilin 1 and watered with high-dose iron served as a model of AD. We evaluated the effects of intranasal administration of the high-affinity iron chelator deferoxamine (DFO) on A\(\beta\) neuropathology and spatial learning and memory deficits created in this AD model. The effects of Fe, DFO, and combined treatments were also evaluated in vitro using SHSY-5Y cells overexpressing the human APP Swedish mutation. In vivo, no significant differences in the brain concentrations of iron, copper, or zinc were found among the treatment groups. We found that high-dose iron (deionized water containing 10 mg/mL FeCl\(_3\)) administered to transgenic mice increased protein expression and phosphorylation of APP\textsubscript{695}, enhanced amyloidogenic APP cleavage and A\(\beta\) deposition, and impaired spatial learning and memory. Chelation of iron via intranasal administration of DFO (200 mg/kg once every other day for 90 days) inhibited iron-induced amyloidogenic APP processing and reversed behavioral alterations. DFO treatment reduced the expression and phosphorylation of APP protein by shifting the processing of APP to the nonamyloidogenic pathway, and the reduction was accompanied by attenuating the A\(\beta\) burden, and then significantly promoted memory retention in APP/PS1 mice. The effects of DFO on iron-induced amyloidogenic APP cleavage were further confirmed in vitro. Collectively, the present data suggest that intranasal DFO treatment may be useful in AD, and amelioration of iron homeostasis is a potential strategy for prevention and treatment of this disease.

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1. Introduction

The predominant neuropathological hallmarks of Alzheimer’s disease (AD) are not only the accumulation of extracellular plaques containing insoluble amyloid-\(\beta\) (A\(\beta\)) peptide and intracellular neurofibrillary tangles (Hardy and Selkoe, 2002; Walsh et al., 2002), but also abnormal enrichment in transition metals such as copper, zinc, and iron (Fe) in the cortical and hippocampal regions of the brain (Bush, 2003; Lovell et al., 1998; Strozyk et al., 2009; Waggoner et al., 1999; Zhang et al., 2010). The A\(\beta\) peptide is generated by \(\beta\)- and \(\gamma\)-secretase cleavages of amyloid precursor protein (APP) (Hardy and Higgins, 1992; Selkoe, 1991). Recent studies have shown that both the proteolytic A\(\beta\) and its precursor APP are associated with metal homeostasis in the AD brain (Atwood et al., 2000, 2003; Bush,
suggesting the possibility that exposure to metals may modulate AD pathology, either by triggering or ameliorating disease progression (Atwood et al., 2000; El Tannir El Tayara et al., 2006; Leskovjan et al., 2011; Robson et al., 2004; Zecca et al., 2004). Fe is a redox-active metal and can provoke oxidative stress through Fenton’s reaction, ultimately causing cell death (Praticò and Delanty, 2000; Sayre et al., 2001). However, recent studies have demonstrated that Fe has a strong binding affinity for Aβ (Atwood et al., 2000; Jiang et al., 2009; Moreira et al., 2010). Furthermore, in the postmortem brains of human AD patients and APP-transgenic mice, Fe was localized in insoluble Aβ plaques (Bush, 2003; Lovell et al., 1998; Smith et al., 1998), and noninvasive magnetic resonance images of living APP-transgenic mouse brains also revealed Fe-enriched Aβ plaques (Zhu et al., 2009). This evidence suggests that the presence of Fe may facilitate the formation of senile plaques (Bush, 2003; Castellani et al., 2004; Lovell et al., 2004).

We recently reported that divalent metal transporter 1 (DMT1) is colocalized with Aβ in the plaques of the human AD brain, and is highly expressed in the cortices and hippocampi of APP/PS1 double transgenic mice (Zheng et al., 2009). Fe influx was reduced by the silencing of endogenous DMT1, which led to reductions in APP expression and Aβ secretion in cells transfected with APP bearing the Swedish mutation (APPsw; characterized by increased Aβ secretion; Zheng et al., 2009). Because Fe is able to promote APP expression via its Fe-responsive 5′-untranslated region (5′ UTR; Mills et al., 2010; Rogers et al., 2002; 2008), and can bind to Aβ peptide (Atwood et al., 2000, 2003; Bush, 2003), it is reasonable to speculate that Fe may be involved in the pathogenic process of AD, and that alteration of Fe homeostasis may provide novel therapeutic strategies against this disease.

Several lines of evidence have demonstrated the neuroprotective effect of metal chelators, which may be applied toward the treatment of AD (Cherny et al., 1999; Crapper McLachlan et al., 1991; Whitnall and Richardson, 2006). One clinical study reported that the Fe chelator deferoxamine (DFO) slowed the clinical progression of cognitive decline associated with AD (Crapper McLachlan et al., 1991). In vitro, DFO is able to inhibit the formation of β-pleated sheets of the Aβ alloform Aβ1–42, and dissolve preformed β-pleated sheets of plaque-like amyloid (House et al., 2004). Similarly, DFO was found to suppress APP holoprotein expression, and to reduce Aβ peptide secretion (Morse et al., 2004). However, while these effects may explain, at least in part, why chelating intracellular Fe levels repress the translation of APP messenger RNA (mRNA) and protect neurons against Aβ toxicity (Avramovich-Tirosh et al., 2007, 2010; House et al., 2004; Morse et al., 2004), the blood–brain barrier is thought to be relatively impermeable to DFO (Shachar et al., 2004). In addition, the long-term clinical use of DFO may lead to systemic metal ion depletion, with subsequent anemia (Cujadungco et al., 2000). Indeed, it has been suggested that clinical treatment with Fe chelators for neurological disorders must be carefully controlled to prevent toxicity and other adverse side effects. Finally, to date there has not been a well-established AD transgenic model that might demonstrate a therapeutic effect for DFO in AD.

In the present study, a mouse model of AD (specifically, APP/PS1 double transgenic mice) was administered noninvasive intranasal DFO to evaluate its effects on AD pathology in terms of Aβ accumulation and cognitive dysfunction. We found that a chronic Fe overload could enhance cognition dysfunction and Aβ accumulation. We also found a high level of Fe-enhanced amyloidogenic APP processing, due to alterations in the expression levels of APP protein and APP cleavage enzymes. These changes were markedly inhibited by intranasally-administered DFO once every other day for 3 months to the transgenic mice. Our data indicate that intranasal DFO treatment reverses Fe-induced memory defects and inhibits amyloidogenic APP processing in APP/PS1 mice.

2. Methods

In brief, the in vivo experimental design consisted of APP/PS1 transgenic mice subjected to a 90-day treatment period (control, dietary Fe, Fe + DFO, or DFO only, n = 6 each) followed by memory and learning tests with a Morris water maze. Blood sera were analyzed for Fe, Cu, and Zn concentrations. The hippocampi and cerebral cortices of the left brain hemispheres were examined for Fe, Cu, and Zn concentrations, protein expression of APP695 and APP threonine (Thr)668, the key enzymes, disintegrin and metalloproteinase domain-containing protein 10 (ADAM10), β-secretase 1 (BACE1), and presenilin 1 (PS1), and APP cleavage fragments secreted form of beta-amyloid precursor protein (sAPP)α, sAPPβ, APP-C83, and C99, as well as mRNA expression of APP. The right hippocampi and cerebral cortices were examined via immunohistochemistry (IHC) for Aβ plaque distribution.

In vitro experiments were performed using human neuroblastoma SH-SY5Y cells stably transfected with human APPsw. Treatments comprised control, FeSO4, DFO, and FeSO4 + DFO; APP protein expression was determined by methyl-thiazolyl-tetrazolium (MTT) assay.

2.1. Transgenic mice and treatments

The male APP/PS1 double transgenic mice used in this study were originally obtained from Jackson Laboratory (West Grove, PA, USA). They were maintained in a controlled environment at standard room temperature and relative humidity, on a 12-hour light/dark cycle with free access to food and water. The Laboratory Animal Ethics Committee of China Medical University approved all experimental procedures.
Mice at the age of 6 months were randomly assigned to 1 of 4 treatment groups (6 mice per group): control, Fe, Fe + DFO, or DFO. The mice in all groups were fed the same standard diet. Mice in the control group were given deionized water ad libitum. Mice in the Fe group were given deionized water containing FeCl₃ (10 mg/mL; Sigma, St. Louis, MO, USA). Those in the Fe + DFO group received Fe-containing water (10 mg/mL), and also intranasally-administered DFO (200 mg/kg body weight, dissolved in saline; Sigma, D9533) once every other day for 3 months. Mice in the DFO group were given water without Fe, and DFO administered as in the Fe + DFO group. The dosages of Fe and DFO were chosen based on previous reports (Hanson et al., 2007; 2009; Unger et al., 2007). The animals’ general health was observed daily and body weights were monitored. Just before decapitation, a whole blood sample was collected directly from the heart, and the serum was separated from the whole blood. The concentrations of Fe, Cu, and Zn in sera and brains were determined using a Siman atomic absorption spectrophotometer (Hitachi 2–200, Tokyo, Japan).

2.2. Morris water maze

Ninety days after the beginning of treatments, the mice were trained and tested in a Morris water maze as previously described (Wang et al., 2010). Briefly, a pretraining test (visible platform training) consisted of 4 trails over 2 days. The mice were given 60 seconds to find the visible platform. Then, the platform was moved to just below the opaque water surface (i.e., making a hidden platform) for the place navigation test, and the animals performed 4 trials per day, with an intertrial interval of 1 minute, for 5 consecutive days. For each trial, their spatial learning scores (the latency period required to escape onto the hidden platform and the path length) were recorded.

On the last day, a 1-minute probe trial was performed with the platform removed. The number of times that each mouse crossed the center of the quadrant where the platform had previously been located was recorded. Finally, the recorded data were analyzed statistically with a computer program (ZH0065; Zhenghua Bioequipments, Yuanyang City, Henan, China).

2.3. Tissue preparation

After the Morris water maze tests, mice were deeply anesthetized with sodium pentobarbital (50 mg/kg intraperitoneal injection) and euthanized by decapitation. The brains were quickly removed and divided into separate hemispheres on an ice-cooled board. The hippocampus and cerebral cortex were dissected from the left hemisphere and kept at −80 °C for Western blot and reverse-transcription (RT)-polymerase chain reaction (PCR) analyses. The right hemisphere was fixed in 4% paraformaldehyde in phosphate-buffered saline at 4 °C overnight and embedded in paraffin, cut into sections (5 μm), and stored at room temperature until stained for morphological analysis.

2.4. IHC and Aβ load measurement

Standard avidin-biotinylated complex IHC staining was performed to analyze the distribution of Aβ plaques in the APP/PS1 mouse brain. Briefly, paraffin sections were dewaxed, rehydrated, and treated in 0.1 M Tris-HCl buffer (Tris-buffered saline, pH 7.4) containing 3% hydrogen peroxide for 10 minutes to reduce endogenous peroxidase activity. After washing with Tris-buffered saline, sections were boiled in citric acid buffer for 3 minutes in a microwave oven. The sections were then rinsed, treated with 5% bovine serum albumin for 30 minutes, and subsequently incubated overnight with mouse anti-Aβ antibody (1:500; Sigma, A5213) at 4 °C. After rinsing, sections were incubated with biotinylated goat anti-mouse IgG (1:200) for 1 hour, followed by streptavidin peroxidase for 1 hour at room temperature. After rinsing, the sections were stained with 0.025% diaminobenzidine for 1 minute. The stained sections were dehydrated, cleared, covered with neutral balsam, and examined with a light microscope equipped with a digital camera (Olympus, Tokyo, Japan). Control sections were treated with identical solutions but without primary antibody followed by all subsequent incubations as described above.

Quantitative image analysis was performed for Aβ IHC by taking micrographs of 5 sections per brain. The number of Aβ-positive plaques in the cortex and hippocampus was counted, and the comparison between vehicle control and the treatment groups was made using Image-Pro plus 6.0 software. Aβ burden was assessed as the percentage of the total area of the cortex and hippocampus that contained regions of Aβ deposits. The data were analyzed with the above-mentioned software.

2.5. Cell culture, drug treatment, and MTT assay

Human neuroblastoma SH-SY5Y cells stably transfected with human APPsw were maintained in Dulbecco’s Modified Eagle’s Medium and Ham’s F-12 nutrient mixture (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-decomplemented fetal calf serum (Gibco), 100 IU/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), and 200 μg/mL G418 (Sigma) at 37 °C in humidified 5% CO₂ air.

Serum-free medium was added for 2 hours when cells reached nearly 70%–80% confluence. Cells were then treated with the medium vehicle, FeSO₄ (100 μM, Sigma), DFO (100 μM), or FeSO₄ + DFO. To ensure that the potential effects of Fe and DFO on APP protein expression were not due to treatment-induced cell death, the concentrations of FeSO₄ and DFO selected were based on previously described studies (Rogers et al., 2002; Zheng et al., 2009) and routine MTT assay. After 24 hours, the cells were collected for Western blot analysis.
2.6. Western blot analyses

Protein extracts from the APP/PS1 transgenic mouse (left) brain tissues and culture cells, and the Western blots, were prepared as described previously (Zheng et al., 2009). Briefly, samples were homogenized in ice-cold lysis buffer containing a mixture of protease inhibitors (10 μg/mL apro- tinin, 10 μg/mL leupeptin, 1 mM NaF, 1 mM Na3VO4 and 1 mM phenylmethylsulfonyl fluoride). After homogenates were centrifuged at 12,000 rpm for 30 minutes at 4 °C, supernatants were collected and the total protein levels were measured using a UV 1700 PharmaSpec ultraviolet spectrophotometer (Shimadzu, Kyoto, Japan).

Proteins (50 μg) were resolved in 8%–15% sodium dodecyl sulfate polyacrylamide gels by electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). After blocking in 5% nonfat milk for 1 hour, the membranes were incubated sequentially overnight at 4 °C with the following primary antibodies: rabbit anti-APP (1:500; Millipore, AB1575); mouse anti-sAPP (1:500; IBL); rabbit anti-PS1 (1:1000; Millipore, AB19026), rabbit anti-BACE1 (1:1000; Sigma, B0681); rabbit anti-APP C-terminal fragments (1:500; IBL, Gunma, Japan); mouse anti-APPα (1:500; IBL, Gunma, Japan); mouse anti-sAPPβ (1:500; IBL); rabbit anti-APP C-terminal fragments (1:4000; Sigma, A8717), and mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:10,000, KC-5G5, Kang Chen, Shanghai, China). After washing, the membranes were incubated with horseradish peroxidase-labeled secondary antibodies (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour.

Blots were visualized using Super Signal West Pico chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL, USA) and Chem Doc XRS with Quantity One software (Bio-Rad, Hercules, CA, USA). The band intensities were quantified using Image-pro plus 6.0 analysis software (Media Cybernetics, Bethesda, MD, USA).

2.7. RT-PCR

Total RNA was extracted from APP/PS1 transgenic mouse brain tissue samples using Trizol (Invitrogen). After quantification by UV-spectroscopy at 260 nm, total RNA of each sample was first transcribed to cDNA using EasyScript Two-Step RT-PCR SuperMix (TransGen Biotech, AE401, Beijing, China). PCR amplification was performed with reagents from TransGen Biotech. The cDNA solution was amplified with primers based on human APP sequences: APP, upstream 5'-GACTGACCACCTGACGAGCTCTG-3', downstream 5'-CTTGAGGATGATCTCTACCCGC-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), upstream 5'-ACGGATTTGGTCGTATTGGG-3', downstream 5'-CGCTTCTGGAGATGTTGAT-3'. The PCR conditions were APP, 35 cycles of 95 °C for 30 seconds, 62 °C for 30 seconds, and 72 °C for 30 seconds; GAPDH, 30 cycles of 95 °C for 45 seconds, 58 °C for 45 seconds, and 72 °C for 60 seconds. The PCR products were normalized with reference to GAPDH mRNA. The results were quantified with ChemDoc XRS Quantity One software.

2.8. Statistical analyses

Repeated measures of analysis of variance (ANOVA) were performed for the Morris water maze tests of latencies and path lengths; differences among means were evaluated with multivariable ANOVA, and results were expressed as the mean ± standard error of the mean (SEM). Other comparisons were analyzed by 2-way ANOVA, and values were expressed as the mean ± standard deviation. All data were analyzed using SPSS 13.0 software (IBM, Armonk, NY, USA), and differences were assumed to be significant if the probability (P) value was < 0.05.

3. Results

3.1. Body weight and metal levels in sera and brains of APP/PS1 transgenic mice

The potential toxicity of long-term treatments with Fe, DFO, or both combined on transgenic mice was evaluated by monitoring their general aspect and body weight. The body weights of mice in both the Fe and Fe + DFO groups were lower than that of either the control (p < 0.01) or DFO groups (p < 0.05; Fig. 1A). No overt signs of toxicity were observed during the entire observation period in the mice of the DFO group. These results were consistent with previous studies, which used the same Fe dosage in the drinking water given to adult C57BL/6 mice (Sobotka et al., 1996; Unger et al., 2007).

To evaluate the effects of the Fe and DFO treatments on APP/PS1 mice, Fe, Cu, and Zn concentrations of both sera and brains were measured 90 days after the start of drug administration. There were no significant differences in the concentrations of any of the metals in brain tissues between the controls and treatment groups, as measured with the atomic absorption spectrophotometer (p > 0.05, for all; Fig. 1B). Notably, although not reaching statistical significance, compared with the control mice a modest increase in Fe levels was observed in those who received the Fe treatment, and limited decreases in Fe and Cu levels were found in the DFO-treated mice, suggesting that intranasal administration may target DFO to the brain as previously described (Hanson et al., 2009).

We detected significantly higher serum Fe levels in the Fe-treated (0.37 ± 0.02 mg/mL), and Fe + DFO-treated groups (0.40 ± 0.03 mg/mL), compared with the controls (0.30 ± 0.02 mg/mL; p < 0.01; Fig. 1C), whereas there were no significant changes in the serum Fe levels of mice treated with DFO alone (0.33 ± 0.05 mg/mL; p > 0.05; Fig. 1C). Levels of serum Cu in the Fe-treated mice and Fe + DFO-treated mice were decreased to 0.51 ± 0.07 μg/mL and 0.33 ± 0.08 μg/mL, compared with that of the controls.
There were no significant changes in the serum Zn levels in any of the treatment groups, compared with controls. We also observed a trend toward increase for Fe and toward decrease for Cu in DFO + Fe-treated mice compared with Fe-treated mice, although the difference did not reach statistical significance. These data suggest that intranasal administration of DFO did not significantly alter Fe, Cu, or Zn levels in the sera of APP/PS1 mice, and imply that intranasal administration delivers very little of the DFO to the blood and peripheral tissues, as previously shown (Hanson et al., 2009).

3.2. Intranasal DFO treatment rescues deficits of memory in APP/PS1 mice

To evaluate whether DFO treatments affect learning and memory in APP/PS1 mice, the mice were subjected to Morris water maze tests 90 days after the start of treatments (Fig. 2). The results of the pretraining, visible platform tests for the Fe, DFO, and Fe + DFO treatment groups were no different from that of the control mice (p > 0.05; Fig. 2A and B), suggesting that neither Fe nor DFO had any significant influence on motility or vision in the transgenic mouse model.

In the place navigation (hidden platform) tests, the mice in the Fe-treated group had a significantly longer escape latency than the control mice (p < 0.05; Fig. 2A). There were no significant differences in path lengths between the Fe group and the control mice (p > 0.05; Fig. 2B). Notably, although the escape latency and the path length of the DFO-treated mice were not statistically different from that of the control mice, there was a tendency toward reduction (p > 0.05 for both; Fig. 2A and B). However, the results of these tests for the DFO-treated mice were significantly decreased compared with the Fe-treated mice (p < 0.05 and p < 0.01, respectively; Fig. 2A and B).

The Fe-treated mice during the probe trial traveled into the center of the quadrant (where the hidden platform had previously been) fewer times than did the control mice (p < 0.05; Fig. 2C). The number of travel times for the DFO-
treated mice was significantly greater than for the controls ($p < 0.01$; Fig. 2C). However, DFO treatment not only significantly promoted learning during the hidden-platform trials, but also significantly improved memory retention during the probe trial ($p < 0.01$; Fig. 2), compared with the Fe treatment.

Collectively, these results suggest that chronic Fe exposure exacerbates deficits of spatial learning and memory, whereas chelation of Fe with intranasally administered DFO not only significantly reversed iron-induced spatial memory impairments but also significantly promoted memory retention compared with the vehicle-treated APP/PS1 mice.

3.3. DFO treatment inhibits Aβ plaque accumulation and reverses Fe-induced Aβ burden in the APP/PS1 mouse brain

The effects of intranasal DFO on Aβ deposition in the APP/PS1 mouse brain were evaluated with IHC (Fig. 3). Fe treatment markedly increased the number and size of Aβ-immunoreactive senile plaques in both the cortex and hippocampus (Fig. 3A). Quantitative analysis showed that the plaque numbers in cortices of the Fe treatment group ($83.8 \pm 5.6$) increased to 175% that of the control ($47.8 \pm 3.2$, $p < 0.01$) and in the hippocampi ($18.6 \pm 1.8$) to 186% that of the control ($10.0 \pm 1.9$, $p < 0.01$; Fig. 3B).

Moreover, we also detected the Aβ burden by measuring the areas of Aβ-positive neuritic plaques in the brain. In the Fe-treated group, the area with Aβ plaques was significantly increased, $206.52 \pm 11.46$% in the cortex and $208.14 \pm 18.93$% in the hippocampus, compared with the control group ($p < 0.01$, for both; Fig. 3B).

However, the cortices and hippocampi of DFO-treated mice showed a significant reduction in plaque number and Aβ burden, compared with either the control or Fe-treated mice (Fig. 3B). There were no differences in plaque number or Aβ burden between the Fe + DFO mice and the controls.

3.4. DFO treatment reduces APP protein level and APP phosphorylation at Thr668 in the APP/PS1 mouse brain

The levels of APP mRNA, and APP protein and phosphorylation of APP at Thr668 ($p$-APP668), were measured by RT-PCR and Western blot, respectively. No differences in the expression levels of APP mRNA were found between the controls and any of the treatments ($p > 0.05$, for all; Fig. 4A). Western blot analyses showed that compared with controls fed a normal diet, treatment with dietary Fe resulted in significantly increased levels of APP695 protein ($132.66 \pm 10.97$%) and phosphorylation ($153.81 \pm 21.96$%; $p < 0.01$, for both; Fig. 4B). In contrast, the expression levels of APP and $p$-APP668 in the DFO group were significantly decreased by $78.85 \pm 6.21$% and $69.84 \pm 10.07$%, respectively, compared with controls ($p < 0.01$, for both; Fig. 4B). In addition, there was a significant reduction in levels of APP protein in the Fe + DFO-treated group compared with mice treated with Fe ($p < 0.01$; Fig. 4B). These results suggest that DFO has a modest inhibitory effect on APP protein expression and activity.

3.5. Regulation of APP processing in APP/PS1 mouse brain via DFO treatment

Given the beneficial role of DFO in APP protein expression and Aβ deposition, we attempted to further determine whether intranasal DFO altered the activities of APP cleavage enzymes in the APP/PS1 mouse brain. Western blot analyses for the relative key enzymes (including ADAM10, BACE1, and PS1) and cleavage fragments of APP (including sAPPα, sAPPβ, C83, and C99) were performed for brain samples of APP/PS1 mice.
Fe treatment significantly reduced the expression level of ADAM10 by 70.75 ± 18.86% compared with the control group (p < 0.05; Fig. 5A), whereas Fe- and DFO-treated mice showed increased levels of ADAM10, by 135.11 ± 26.77% (p < 0.05) and 195.59 ± 20.13% (p < 0.01), respectively (Fig. 5A). In contrast, the levels of BACE1 were significantly increased in the Fe-treated mouse brain by 118.25 ± 9.34% (p < 0.01; Fig. 5A), but decreased in the DFO-treated by 76.88 ± 5.91% (p < 0.01; Fig. 5A). The levels of PS1 in the Fe- and Fe + DFO-treated mouse brain were significantly increased by 146.04 ± 19.51% (p < 0.01) and 123.12 ± 16.60% (p < 0.05), respectively, but decreased in the DFO-treated by 68.77 ± 6.78% compared with the control group (p < 0.01; Fig. 5A).

For an appraisal of the potential therapeutic effect of DFO in AD, the levels of α-secretase-generated sAPPα/C83 and β-secretase-generated sAPPβ/C99 fragments were examined in the transgenic mouse brain. The levels of sAPPα were not notably different between the control and Fe-treated transgenic mice (p > 0.05; Fig. 5B), but the level of sAPPβ in the Fe group increased 157.99 ± 16.63% (p < 0.01; Fig. 5B). The level of sAPPα in the DFO-treated mouse brain was increased to 159.43 ± 13.95% of the control (p < 0.01; Fig. 5B), and that of sAPPβ was reduced to 67.37 ± 17.68% of the control (p < 0.05; Fig. 5B).

Further, the levels of C99 were increased to 144.99 ± 15.86% of the controls in the brains of Fe-treated mice (p < 0.01; Fig. 5B), and the levels of C83 fragments were increased to 196.86 ± 6.92% in DFO-treated mice (p < 0.01; Fig. 5B).
Therefore, we can infer that in the brains of the Fe-treated transgenic mice /H9251-secretase cleavage activity was markedly decreased, while /H9252- and /H9253-secretase were increased. The converse was found in DFO-treated mice, suggesting that DFO promoted nonamyloidogenic processing of APP and attenuated cerebral amyloidosis in the APP/PS1 mouse brain.

3.6. DFO treatment affects expression levels of APP cleavage enzymes and products in APPsw-transfected cells

The findings in APP/PS1 mouse brains presented above prompted us to use human neuroblastoma SH-SY5Y cells stably transfected with human APPsw to investigate further the roles of high Fe exposure and DFO-treatment in APP processing and A\beta secretion. We selected 100 \mu M FeSO4 and 100 \mu M DFO as optimal concentrations, based on an evaluation of cell viability with an MTT assay (Fig. 6A and B).

Western blot analysis showed that high Fe (100 \mu M) exposure significantly increased the levels of the APP cleavage enzyme BACE1 to 150.07 \pm 35.05\%, and PS1 to 182.77 \pm 11.82\%, of untreated controls (p < 0.05, for both; Fig. 7B). Additionally, the levels of the \beta-secretase-generated fragments sAPP\beta and C99 were markedly increased to 140.49 \pm 16.12\% and 172.58 \pm 32.64\%, respectively, (p < 0.01). No changes were observed in the levels of ADAM10 and \alpha-secretase-generated sAPP\alpha (Fig. 7B).

By contrast, chelation of Fe with 100 \mu M DFO increased the expression levels of ADAM10 and sAPP\alpha to 221.20 \pm 23.12\% (p < 0.01; Fig. 7A) and 163.90 \pm 12.86\% (Fig. 7B, p < 0.01), respectively, and reversed the changes in expression levels of the cleavage enzymes and \beta-secretase-generated fragments of APP induced by high Fe exposure (Fig. 7B). Thus, it is likely that DFO suppresses the amyloidogenic APP cleavage pathway and A\beta generation by competing for binding to Fe in APPsw cells.

4. Discussion

DFO is a Fe chelator that has been approved by the Federal Drug Administration for use in iron-overload disease to promote the excretion of excess Fe. In many neurological disorders Fe accumulation in the brain is involved in neuronal death, and thus several studies have pointed out that DFO could be a candidate for use in AD (Craper McLachlan et al., 1991; Hanson et al., 2007; House et al., 2004; Morse et al., 2004; Savory et al., 1998). However, problems associated with systemic delivery of DFO have limited its utility in this regard. Such problems include neurotoxicity and systemic metal ion depletion after long-term treatment (Cuajungco et al., 2000; Freedman et al., 1988; Lindnér et al., 1995), poor gastrointestinal absorption and rapid degradation (May and Bulman, 1983), and difficulty crossing the blood–brain barrier (Shachar et al., 2004).

Interestingly, several studies have shown that drugs delivered intranasally could target the brain along the olfactory and trigeminal nerves, bypassing the blood–brain barrier (Frey et al., 1997). Furthermore, a recent study

Fig. 4. Expression levels of APP mRNA and amyloid precursor protein (APP) in deferoxamine (DFO)-treated APP/PS1 mouse brain. (A) Reverse-transcription (RT)-polymerase chain reaction (PCR) showed that there was no difference in APP mRNA levels in the transgenic mouse brain between control, Fe-, Fe + DFO-, and DFO-treated mice. (B) Western blot analysis revealed that Fe treatment significantly increased the level of APP695 and phosphorylation of APP at threonine (Thr)668 (p-APP668) protein, whereas DFO treatment significantly reduced APP695 and p-APP668 protein levels, compared with controls. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was the internal control. Each group, n = 6; * p < 0.05, ** p < 0.01 compared with the control group; # p < 0.05, ## p < 0.01 compared with the Fe treatment group (2-way analysis of variance).
demonstrated that compared with intravenous administration, intranasal treatment with DFO increased targeting to the frontal cortex by 271-fold (Hanson et al., 2009). In the current study, APP/PS1 mice treated with intranasally-administered DFO exhibited a reduction in Fe-induced memory impairments, based on their performance during the Morris water maze tests. Subsequent to these tests, the brains of these mice were examined for Aβ amyloidosis and APP expression and cleavage.

It is not well understood from the results of these studies how DFO, acting in the brain, provided significant protection from AD. The current study focused on demonstrating an association between Fe and Aβ deposition, and then clarifying whether intranasal DFO treatment inhibits Aβ toxicity via the modulation of Aβ-metal interactions. To explore this possibility we evaluated the concentrations of iron, copper, and zinc in the brain with reference to the control mice. Consistent with previous reports (Sobotka et al., 1996; Unger et al., 2007), we found no significant augmentation of Fe concentrations in the brains of the mouse model following a high intake of dietary Fe, although the levels of serum Fe were significantly elevated. These findings indicate that systemic Fe overload has minimal effect on central nervous system Fe content. However, subsequent chelation of Fe via intranasal DFO treatment further elevated the levels of serum Fe, suggesting that intranasal administration of DFO does not deliver much DFO to peripheral tissues and chelate unbound iron (Hanson et al., 2009). Furthermore, it is worth noting that dietary Fe modestly increased the levels of Fe in the brain and the treatment with DFO attenuated these alterations, suggesting that intranasal administration targeted DFO to the brains of the APP/PS1 mice.

In contrast, we found that iron overload resulted in a trend toward decrease in serum copper levels, and this decrease was markedly enhanced after the DFO treatment. These results resemble the observations of previous studies in which serum copper levels were lower in iron-loaded rats (Crowe and Morgan, 1996; Yu et al., 1994) and humans (Newhouse et al., 1993). Our results are further supported by a report that treatment consisting of Cu alone reduced serum iron levels in APP751SL transgenic mice (Schäfer et
The reasons for the changes in copper levels after Fe or Fe\textsubscript{II}/H\textsubscript{11001}DFO treatment in the present study are unclear, but could be because Fe and copper uptake are physiologically linked (Fox, 2003) by competing DMT1 for absorption (Gunshin et al., 1997). Of course, DFO selectively binds multiple metals including aluminum, copper, zinc, and chromium (Bush and Tanzi, 2008; House et al., 2004; Molina-Jijón et al., 2012), although it is a specific iron chelator. Thus, in the present study the reduced level of serum copper in the mice treated with Fe\textsubscript{II}/H\textsubscript{11001}DFO was caused, at least partly, by the iron overload and the chelation of DFO. Nevertheless, we observed no significant changes in the zinc levels of either brains or sera after chronic Fe, DFO, or Fe\textsubscript{II}/H\textsubscript{11001}DFO treatment.

Although we did not see a significant difference between the DFO treatment group and the control mice in the total content of metals in either sera or brain tissues, a modest decrease in the concentrations of Fe and Cu were detected. It may be that the DFO treatment caused the chelation of the small fraction of nonprotein bound iron in the sera and brain tissues (Bush and Tanzi, 2008). However, while it cannot be excluded that the ability of DFO to chelate metals may have had a role in the protective effects observed in our study, chelation is involved in the formation of a metal complex that cannot readily cross the blood–brain barrier (Crowe and Morgan, 1994).

It is interesting that clioquinol was found to attenuate APP/A\textbeta pathology by mediating the redistribution of copper (by facilitating copper uptake into the cell), rather than by removing copper from the system (Cherny et al., 2001; Treiber et al., 2004). As an aside, it is not surprising that intranasal treatment of DFO normalized serum Fe concentration, given that DFO is rapidly delivered to the brain along the olfactory and trigeminal nerves with negligible adverse systemic side effects. Therefore, we conjecture that mechanisms other than chelated Fe are probably responsible for the neuroprotective functions of DFO. It has been found that DFO blocks the production of reactive oxygen species (ROS) by inhibiting iron’s catalytic role (via Fenton’s reaction; Chaston and Richardson, 2003; De Lima et al., 2008; Winterbourn, 1995). The suppression of reactive oxygen species subsequently modulates the gene expressions of hypoxia-inducible factor (HIF1A), iron regulatory protein-1 (IRP1), and APP (Cho et al., 2010; De Lima et al., 2008; Hamrick et al., 2005; Venti et al., 2004) thus decelerating AD progression. Further research efforts into how DFO administered intranasally affects APP gene expression and its posttranslational modifications are crucial to the understanding of this drug’s potential efficacy in treating AD.

More and more evidence supports the involvement and interrelatedness of cortical and hippocampal Fe dysregulation, APP synthesis and processing, and A\textbeta amyloidosis in the pathogenesis of AD (Bandyopadhyay et al., 2006; Lee et al., 2003; Rogers and Lahir, 2004). The 5’ UTR of APP mRNA has a functional element (Rogers et al., 2002) that is selectively responsive to the presence of Fe, so that intracellular APP synthesis is regulated by intracellular Fe levels in a feedback pattern. Studies with neuroblastoma cells in vitro have shown that Fe treatment promotes the expression of APP via its 5’ UTR (Rogers et al., 2002; 2008), whereas Fe chelation with DFO represses APP translation (Perez et al., 2010). This suggests that Fe levels have an important role in the expression of APP protein. Moreover, the phosphorylated modification of APP protein prior to proteolysis, in particular at site Thr668, may promote the production of A\textbeta (Maynard et al., 2009). However, although there have been some in vitro studies that tested the effects of Fe on APP processing (Perez et al., 2010; Rogers et al., 2002), there have been no prior detailed reports that used transgenic animal models of AD to investigate whether Fe binding to APP alters APP processing and A\textbeta production in vivo.

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**Fig. 6.** Cell viability in APP\textsuperscript{sw} cells treated with Fe and deferoxamine (DFO). (A, B) Methyl-thiazolyl-tetrazolium (MTT) analyses were performed on SH-SY5Y cells stably transfected with human APP\textsuperscript{sw}, to select appropriate concentrations of FeSO\textsubscript{4} and DFO for the in vitro studies. The cells were treated with indicated concentrations of FeSO\textsubscript{4} and DFO for 24 hours. Based on cell viability, 100 \mu M FeSO\textsubscript{4} and 100 \mu M DFO were selected for the Fe treatment and Fe chelation experiments, respectively.
In the present study, a high intake of dietary Fe in vivo significantly augmented the expression levels of APP protein in the APP/PS1 transgenic mouse brain, whereas intra-nasal DFO treatment significantly reduced levels of APP695 protein. In addition, we found that Fe treatment in mice stimulated the phosphorylation of the Thr668 site in APP, whereas the p-APP668 expression levels in DFO-treated mice were significantly reduced compared with the Fe treatment group. Taken together, the results of this study indicate that intranasal DFO reduces Fe-induced Aβ deposition, which occurs, at least partly, through reduction of the expression and phosphorylation of APP protein in the APP/PS1 mouse brain.

In the normal brain, APP protein is predominantly cleaved through the nonamyloidogenic β-secretase pathway (Esch et al., 1990). However, in the brain with AD, APP is cleaved through the amyloidogenic β-secretase pathway to form neurotoxic Aβ, which is involved in AD pathogenesis (Bodles and Barger, 2005; Haass et al., 1992; Shoji et al., 1992). The present study found that within the transgenic mouse brain and APP695-overexpressing cells, Fe treatment promoted the amyloidogenic APP cleavage pathway via elevated levels of BACE1 and PS1. On the other hand, DFO reversed Fe-induced Aβ production by way of increased ADAM10 and reduced BACE1 and PS1 levels, resulting in decreased secretion of sAPPβ compared with sAPPα. Furthermore, IHC results showed a reduction in Aβ burden in the DFO-treated APP/PS1 mouse brain. Thus, the data suggest that DFO is able to shift the processing of APP to the nonamyloidogenic pathway.

In summary, the present study demonstrated that the Fe chelator DFO can reverse Fe-induced recognition memory deficits and inhibit amyloidogenic APP processing in the brains of APP/PS1 transgenic mice. It also directly increases passing times on the Morris water maze, decreases plaque numbers and burden, decreases the levels of APP expression and phosphorylation, and modulates the processing of APP to the nonamyloidogenic pathway.
Table 1
Overview of changes of the property being compared in the APP/PS1 mice after DFO and/or Fe treatment

<table>
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<tr>
<th></th>
<th>Fe</th>
<th>Fe + DFO</th>
<th>DFO</th>
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<tr>
<td>Body weight</td>
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<tr>
<td>Brain metal concentrations</td>
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<td>Cu</td>
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<tr>
<td>Zn</td>
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<tr>
<td>Serum metal concentrations</td>
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<tr>
<td>Fe</td>
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<td>Zn</td>
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<tr>
<td>Morris water maze</td>
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<td>Escape latency</td>
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<td>Passing times</td>
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<td>APP levels</td>
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<tr>
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<td>↑↑</td>
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<tr>
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<tr>
<td>ADAM10</td>
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<td>BACE1</td>
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<td>PS1</td>
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<tr>
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<td>C99</td>
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Changes are shown with arrows: ↑ significant increase; ↓ significant decrease; → nonsignificant. Each group, n = 6; a single arrow represents p < 0.05, double arrows represent p < 0.01 compared with the control group.

Key: Aβ, amyloid beta; ADAM10, metalloproteinase domain-containing protein 10; APP, amyloid precursor protein; BACE1, β-secretase 1; DFO, deferoxamine; mRNA, ; p-APP668, phosphorylation of APP at threonine 668; PS1, presenilin 1; sAPP, .

genic pathway via increasing and decreasing various APP-cleavage related enzymes and other proteins in the APP/PS1 transgenic mice (Table 1). The present data, together with previous reports (Crapper McLachlan et al., 1991; Hanson et al., 2007; House et al., 2004; Morse et al., 2004; Savory et al., 1998), suggest that intranasal administration of DFO may be a safe and noninvasive therapeutic strategy for AD therapy.

Disclosure statement

The authors declare that there are no competing interests.

All experimental procedures were approved by the laboratory animal ethical committee of China Medical University.

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